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Uptake of Bacteriophage f2 through Plant Roots

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16. ABSTRACT

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Uptake of Bacteriophage f2 Through Plant Roots

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A model system was designed to measure viral uptake through the roots of plants and translocation to distal plant parts. For this study, uptake of bacteriophage f2 was measured in corn and bean plants growing in hydroponic solutions. Few phage were detected in plants with uncut roots. However, when roots of both plant types were cut just before exposure to very high concentrations of phage, the amount of phage uptake was several orders of magnitude greater than with uncut roots, but still was considerably less than that which was theoretically possible. Furthermore, cut roots were rapidly repaired, thus inhibiting uptake, and the amount of uptake in plants with cut roots was proportional to phage exposure levels. Finally, phage were transported to all plant parts examined, but their survival times within each portion of the plants appeared to be of limited duration. All of these factors tend to minimize the possible public health significance associated with viral uptake through the root systems of plants.

It has been reported that viruses can persist for many days on the surfaces of plants (4, 10). Little is known about viruses that reach the interior of plants, but these may have even longer survival times. The most obvious route for a virus to follow from soil or hydroponic solution to the interior of a plant is through the plant root system.

It has been suggested that viruses cannot penetrate the intact cuticle or epidermis of a plant root (5). However, breaks in this protective coating could allow viral penetration. Root damage by soil fungi or nematodes has been reported to be responsible for the transmission of plant viruses in a number of instances (5, 9). Other causes of root damage, including that sustained during transplantation or cultivation, can easily be visualized.

Once a virus enters a plant there is ample evidence that it can move rather rapidly through the phloem to distal parts of the plant (1–3). In this way a human virus taken up through the roots could potentially contaminate all portions of the plant. Although the evidence is rather limited, several reports suggest that human and other animal viruses can enter through roots and be translocated to the leafy portions of plants (6, 7, 11).

Because of the possible significance of these findings regarding waste utilization and its effects on human health, a study was designed to quantify, under ideal conditions, the uptake of viruses through roots and movements into upper parts of plants. Representative monocotyledon (corn) and dicotyledon (bean) plants were chosen for this study. The bacteriophage f2 was used as the model virus because of its small size, its structural similarity to human enteric viruses, and its ability to be easily produced in high concentrations. To maximize viral uptake, plants were grown in hydroponic solutions to which high concentrations of f2 were added. Uptake was further maximized by cutting the roots of a portion of the plants. The results of this study are reported here.

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MATERIALS AND METHODS

Bacteriophage growth and infectivity assay. The bacterial virus f2 used in this study was grown and measured for infectivity by the plaque assay in *Escherichia coli* A-19. The bacterial growth medium contained 10 g of tryptone, 8 g of NaCl, 1 g of yeast extract, 1 g of dextrose, and 0.3 ml of 6 N NaOH per liter. A double agar procedure was used for the plaque assay. The bottom agar contained 10 g of tryptone, 10 g of agar, 2.5 g of NaCl, 2.5 g of KCl, and 1 ml of 1 M $CaCl_2$ per liter, and the top agar contained 10 g of tryptone, 6 g of agar, 8 g of NaCl, 1 g of dextrose, and 1 g of yeast extract per liter. The bottom agar (15 ml) was poured into 100-mm plates and allowed to harden. Melted top agar (3 ml) at 45°C was mixed with 1 ml or less of phage (diluted in nutrient broth where appropriate) and 0.1 ml of a fresh culture of A-19, immediately poured over the bottom agar, and allowed to harden. The plates were inverted and incubated at 37°C for 7 h before plaques were enumerated.

Plant growth. The corn (*Zea mays* L.) and bean (*Phaseolus vulgaris* L.) plants used in this study were grown hydroponically at 22°C in nutrient solutions (NO_3 [11 meq/liter], SO_4 [3 meq/liter], H_2PO_4 [2 meq/liter], Ca [8 meq/liter], Mg [3 meq/liter], K [5 meq/

liter], B [0.5 ppm; 0.5 μg/ml], Mn [0.5 ppm], Zn [0.05 ppm], Cu [0.01 ppm], Mo [0.01 ppm], and Fe [4 ppm]) (8) under conditions of artificial lighting. Plants were grown individually in plastic cups with apertures cut in the bottom to permit passage of roots. These cups were set into holes in the lids of buckets (six plants per bucket) each containing 12 liters of nutrient solution. Each lid contained an additional hole for supplementing or sampling the nutrient solution. This solution was replenished daily until the time of phage addition, and its pH was maintained between 5.0 and 5.5 by adding either KNO_3 or KOH. The plant roots were kept continuously aerated to allow maximum oxygen uptake and plant growth. Both plant species were grown for approximately 3 weeks after germination before the addition of phage.

Exposure of roots to phage. To prevent root damage in plants before phage addition, the only manipulation performed was replenishment of water and nutrients in the buckets. Immediately before the addition of phage, the roots of all the plants in certain buckets were severed at about their midpoints (20 to 30 cm from the original point of growth). One plant per bucket was removed immediately and processed as a control. A lysate of bacteriophage f2 containing the numbers of phage prescribed for each experiment was then added (100 ml per bucket) to specified buckets in the absence of aeration. The hole in the lid through which the phage was added was then sealed, and aeration was continued. Further discontinuities in aeration occurred only when the nutrient solution was being sampled. One-third of the buckets did not receive phage in these experiments and served as controls. These buckets were randomly interspersed with the others to detect general viral contamination.

Harvesting, processing, and analysis of plants. To measure uptake of phage, the stems of plants were severed 10 cm above the original point of growth (about 20 cm above the nutrient solutions), and the entire upper portions of the plants were analyzed unless stated otherwise. The wet weights of each plant were determined, and appropriate volumes of nutrient broth were added. The mixtures were blended for 1 min at high speed, and the samples were frozen and stored at −20°C. Viable phage determinations of these samples were then made by the plaque assay. For this, thawed samples were diluted in nutrient broth (where necessary), and 1-ml volumes were analyzed for recoverable PFU. Finally, these values were related to the original weight of plant material, and the PFU per wet weight of plant tissue was calculated.

RESULTS

Preliminary experiments. Before the measurement of uptake of bacteriophage f2 into plants, several factors regarding the detectability and stability of f2 had to be determined. The first factor was the effect of plant extracts on the plaquing efficiency of the phage. For this, samples of f2 in nutrient broth (0.2 ml) with about 100 PFU were mixed with 1-ml volumes of blended corn or bean suspensions containing 1 part plant tissue and 2 parts nutrient broth. The plaquing efficiencies of the phage were then measured relative to samples not containing the plant extract. In three separate experiments, recoveries of 73, 67, and 71% were made from corn extracts, and recoveries of 65, 95, and 107% were made from bean extracts. Therefore, the plant extracts themselves had little effect on the plaquing efficiencies of this phage.

The second preliminary factor to be determined was possible toxic effects of the plants on f2. To permit what would appear to be optimal conditions for the expression of toxicity, f2 was mixed with blended suspensions of both corn and bean plants and incubated at room temperature for a 2-week period. The decrease in phage recovery was comparable to that found in a control sample of phosphate-buffered saline (data not shown). Therefore, it appears that neither plant is unusually toxic for this phage.

The final preliminary factor to be tested was the stability of f2 in the nutrient solution used to support plant growth. Because this solution would be continuously aerated during actual experimentation, f2 stability was measured at room temperature under aerobic conditions. A decrease in phage recovery of only about 0.5 order of magnitude was observed after 8 days (data not shown). Similar rates of phage inactivation were found in nutrient solution in later experiments.

Viral uptake by plants. (i) Intact versus cut roots. After demonstration that the experimental protocol planned for this study was satisfactory, an initial uptake experiment was performed. For this, nine buckets of each plant type (six plants per bucket) were grown in hydroponic solution. Three buckets of each plant received no phage. These were interspersed as controls among three buckets with intact roots and three buckets with cut roots, all receiving phage (final concentration, 10^{10} PFU/ml). One plant from each bucket was harvested and analyzed either just before phage addition or at 1, 2, 3, 4, and 7 days after phage was added. Therefore, three replicates were analyzed at each time point for every treatment.

No uptake of phage through intact roots of corn plants was found until day 4 after phage was added, and then the amount recovered was not much above the limit of detection (Fig. 1). No further increase was observed by day 7. However, a large number of phage were taken up and transported in corn plants with cut roots, even within 1 day. Again, maximal numbers of phage were found on day 4. A similar result was noted with bean plants, except that maximal uptake was attained more rapidly, and the total amount of phage recovered relative to plant weight was greater (Fig. 2). Of 36 control plants, 1 was found to be contaminated. Although this is significant, the consistency of the results found with other samples diminishes the possibility

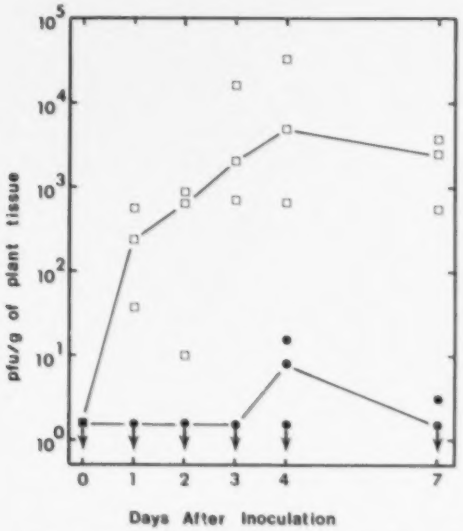

FIG. 1. Uptake of f2 into the stalk of corn plants with cut (□) versus uncut (●) roots. The experimental protocol is presented in the text. The arrows indicate values that are below the limit of detection of 1.5 PFU/g of plant material. The lines are drawn to connect the median of the three replicate values obtained at each point in time.

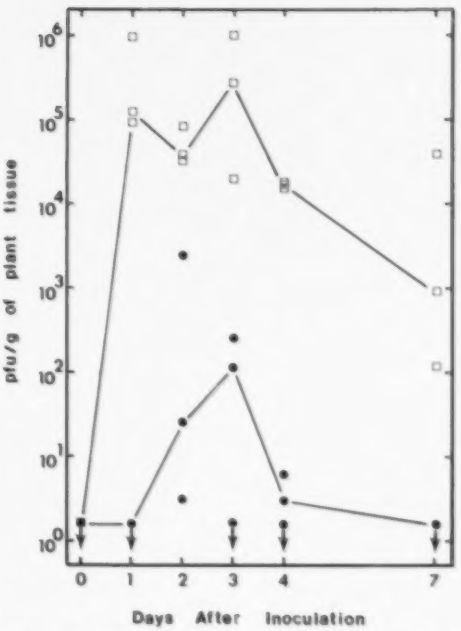

FIG. 2. Uptake of f2 into the stem and leaves of bean plants with cut (□) versus uncut (●) roots.

that phage detected in any of these samples was due merely to contamination.

These results show that plants with intact roots allow little phage uptake and transport. However, plants with cut roots readily take up f2. Because in nature roots can be easily damaged, uptake into plants with cut roots was examined in greater detail.

(ii) Dose dependency. Although uptake into plants with cut roots was very significant, the number of phage detected was very small relative to the exposure level. It was possible that this was a maximal number of phage that could be assimilated, and this limit can be reached at much lower exposure levels. If so, then it was also possible that similar numbers of viruses could be taken up by plants with damaged roots at quite low exposure levels.

To test the relationship between exposure level and uptake, only bean plants with cut roots were studied. For this experiment, uptake into plants was compared at three viral exposure levels: the high level of the previous experiment (1×10^{10} PFU/ml) and two lower levels (5×10^{7} and 5×10^{5} PFU/ml). As in the previous experiment, three replicates were analyzed at each point in time. The results are plotted to demonstrate the relationship between phage uptake and exposure level as a function of time after exposure (Fig. 3). The amount of uptake per unit dose at all three exposure levels can be fitted to a single straight line whose slope decreases as a function of time. If it is assumed that this straight line represents the values of uptake per unit dose at the high dose (10^{10} PFU/ml), then hypothetical lines can be drawn parallel to this line which represent the amount of uptake per unit dose expected at lower exposure levels if uptake were independent of dose. These lines are A (dose, 5×10^{7} PFU/ml) and B (dose, 5×10^{5} PFU/ml). Clearly, the actual uptake per unit dose values are much closer to the original straight line than to the hypothetical lines. Therefore, the results indicate that viral uptake is highly dose dependent. Although this was the result anticipated, experimental demonstration was extremely important because of the theme of this study.

(iii) Rate of translocation and distribution of phage within plants. In the previous experiments the entire plant 10 cm or more above the point of initial growth was analyzed for phage. It was possible that viruses never went much beyond this 10-cm point. Therefore, an experiment was designed to determine the distribution of phage within plants and how rapidly the final distribution is attained.

The experimental design was to expose three

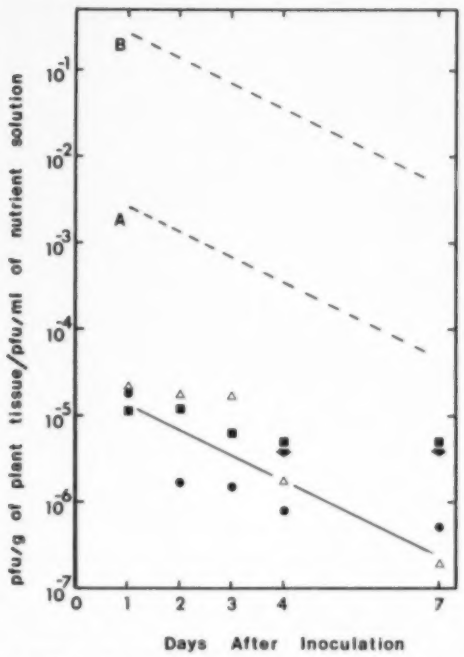

FIG. 3. Recoveries of f2 in bean plants as a function of viral exposure level. The roots of bean plants growing in hydroponic solution were cut near their midpoints and immediately exposed to either 1×10^{10} (△), 5×10^{7} (●), or 5×10^{5} (■) PFU of bacteriophage f2 per ml of nutrient solution. Uptake into the stem and leaves was determined at specified times after phage addition. The values given represent the average of three replicates for every exposure level at each point in time. The dashed lines represent hypothetical values of uptake per unit dose expected if uptake at exposure levels of 5×10^{7} PFU/ml (A) and 5×10^{5} PFU/ml (B) were independent of dose. Arrows indicate values that are below the limit of detection for this study.

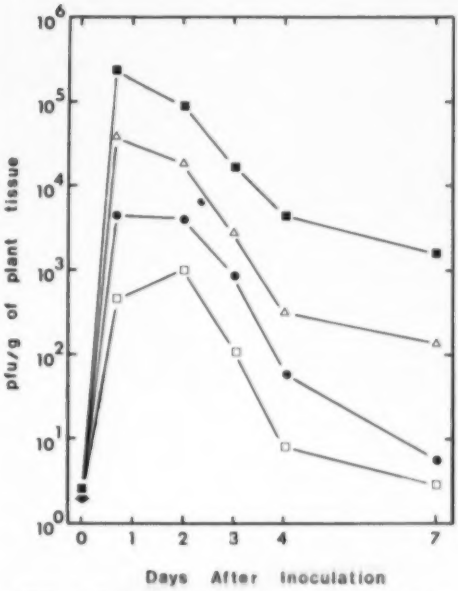

FIG. 4. Recovery of f2 from different parts of bean plants. The protocol for this experiment is presented in the text. The values represent the averages for three replicates at each point in time. Plant parts: lower stem (■), upper stem (△), upper leaves (●), lower leaves (□).

buckets of bean plants with cut roots to a high concentration of phage (5×10^{9} PFU/ml of nutrient solution) and analyze one plant from each bucket at every time point (three replicates per time point). In this experiment, however, harvested plants were also severed at points midway up their stems from the initial cuts. The lower and upper leaves along with the lower and upper stems were separately analyzed for the presence of phage. To insure that phage found in leaves was truly in the leaf blades, the petioles were carefully cut from each leaf.

Uptake and transport of phage was rapid because nearly maximal levels were detected in all stems and leaf blades by 16 h after exposure (Fig. 4). However, a distinct gradient effect was found for viral distribution within the plant. Furthermore, in both this and in previous experiments viral numbers were found to decrease rather rapidly in bean plants (2 to 3 orders of magnitude per week). This effect is presumed to be due to viral inactivation.

(iv) Rate of root repair. In all experiments described until now, plants were exposed to phage within minutes after their roots were severed. It is likely that root damage can be repaired which would block virus uptake. To determine how fast this occurs, roots of bean plants in four buckets were severed. Phage was then added to one bucket immediately and at 1, 3, or 7 days to each of the other buckets. Three plants from each bucket were harvested 1 day after phage addition, and the other three plants were harvested 7 days after phage addition.

Virus uptake decreased quite rapidly with time of root repair (Fig. 5). On the basis of these results, repair is nearly 99% complete within 3 days. The rate of repair is comparable whether measured 1 day or 7 days after phage addition.

DISCUSSION

It has been reported that human and animal viruses can be taken into plants through their

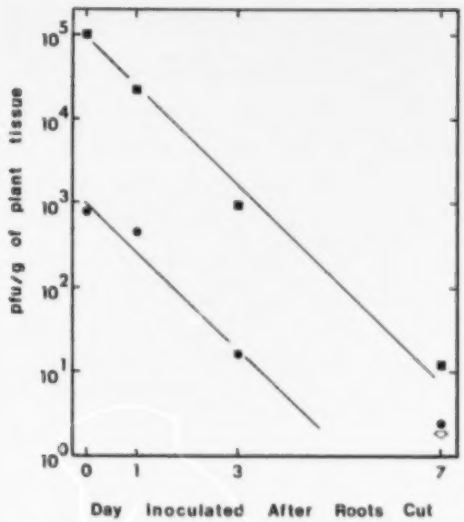

FIG. 5. Effect of repair time on uptake of f2 through cut roots of bean plants. The protocol for this experiment is presented in the text. The values given are the averages of three replicates assayed under each condition at either 1 day (■) or 7 days (●) after phage addition.

root systems (6, 7, 11), but neither the circumstances associated with this event nor the probability of its occurrence has been defined. The purpose of the present study was to determine the extent of viral uptake through plant roots under optimum conditions and the effect on uptake that occurs when certain of these conditions are modified.

Uptake of the bacteriophage f2 through the cut roots of corn and bean plants in hydroponic solution was consistently large as measured through analysis of the upper parts of these plants. However, the amount of phage detected in these upper plant parts was much less than was theoretically possible if all of the water that entered and was distributed throughout plants after phage exposure retained the exposure level of phage. Maximal phage concentrations of about 10^6 PFU/g of plant tissue were detected in plants whose roots were exposed to 10^{10} PFU/ml in nutrient solution. Conceivably this 10^{-4}-fold difference in concentration was partially due to a reduced ability to detect viable phage within plant tissues. The magnitude of this effect should be minimal, however, because plant extracts of both corn and beans had little influence on the plaquing efficiency of f2. It is more likely that the interiors of plants act as molecular sieves and permit only a portion of the phage to be moved from one barrier to the next. Evidence for this effect is provided by the observed differences in viral recoveries from the different parts of bean plants (Fig. 4).

The amount of uptake into plants with uncut roots was several orders of magnitude less than that into plants with cut roots. Although great care was taken to protect uncut roots in this study, the small amount of uptake observed could have been due to a limited amount of root damage. It is also possible that portions of uncut roots, such as their growing tips, are more susceptible to viral penetration. In any event it appears that undamaged roots are quite resistant to viral penetration, even in hydroponic solution where the maximal viral uptake is expected.

Although viral uptake could occur in damaged roots under conditions found in nature, the possibility of uptake should be transient because of root repair. The repair rate observed in the laboratory experiments reported here (Fig. 5) may be more rapid than normally occurs under field conditions. Even so, root repair should be a significant deterrent to viral uptake.

The stability of viruses within plants is another major factor that influences the possible health hazard associated with virus uptake. This stability will depend on environmental conditions as well as the particular virus-plant system under consideration. The decreased recovery of f2 in bean plants as a function of time (Fig. 2, 3, and 4) probably reflects the minimum die-off rate of this virus in these plants under a particular set of experimental conditions. Because viral uptake into these plants is rapidly inhibited by root repair, the die-off rate observed may closely approximate the real rate for this system. Unfortunately, it is impossible to predict from these results the stability of any human virus in this or any other plant system. However, because of the structural similarities between f2 and human enteric viruses, their stabilities in plants may be comparable.

It would be of much value if the results shown here could be used directly to help quantitate the human health hazard associated with the growth of food crops in sewage products. This is not possible. The results do show, however, that the risk of viral uptake through plant roots and subsequent transport to distal parts of plants cannot be great if plant-virus systems in nature behave like the model system studied here. The small amount of uptake relative to exposure levels in plants with intact roots, the dose-response relationship between exposure level and uptake, and the rate of root repair all tend to minimize this risk. Formation of stable associations between plants and viruses by more direct routes, such as spray irrigation of sewage onto growing crops, may represent a greater threat to human health.

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